Accumulation of wound-inducible ACC synthase transcript in tomato fruit is inhibited by salicylic acid and polyamines

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Abstract

Regulation of wound-inducible 1-aminocyclopropane-1-carboxylic acid (ACC) synthase expression was studied in tomato fruit (*Lycopersicon esculentum* cv. Pik-Red). A 70 base oligonucleotide probe homologous to published ACC synthase cDNA sequences was successfully used to identify and analyze regulation of a wound-inducible transcript. The 1.8 kb ACC synthase transcript increased upon wounding the fruit as well as during fruit ripening. Salicylic acid, an inhibitor of wound-responsive genes in tomato, inhibited the wound-induced accumulation of the ACC synthase transcript. Further, polyamines (putrescine, spermidine and spermine) that have anti-senescence properties and have been shown to inhibit the development of ACC synthase activity, inhibited the accumulation of the wound-inducible ACC synthase transcript. The inhibition by spermine was greater than that caused by putrescine or spermidine. The transcript level of a wound-repressible glycine-rich protein gene and that of the constitutively expressed rRNA were not affected as markedly by either salicylic acid or polyamines. These data suggest that salicylic acid and polyamines may specifically regulate ethylene biosynthesis at the level of ACC synthase transcript accumulation.

Introduction

Fruit ripening is a late stage of fruit tissue differentiation which precedes tissue senescence [8, 17, 39]. A myriad of biochemical and anatomical changes accompany the ripening process. Ethylene has been established as the major plant hormone promoting fruit ripening [8, 22, 29]. Ethylene promotes seed germination [49], leaf senescence [27], flower abscission [44] and development of plant defense systems [7]. On the other hand, ethylene inhibits cell division and cell

differentiation [2, 22, 50]. Furthermore, ethylene interacts with other plant growth regulators such as auxin [9], polyamines [3, 27, 38, 48] and salicylic acid [21] thereby altering the physiology of plants. Understandably, therefore, controlling ethylene biosynthesis and action is of considerable interest to plant biologists. Deciphering the regulatory controls that initiate the process of fruit ripening should provide important information that is fundamental to this aspect of tissue differentiation.

In higher plants, ethylene biosynthesis proceeds

mostly via the metabolic pathway, methionine → S-adenosylmethionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene [1]. The rate-limiting step in the production of ethylene is the conversion of SAM to ACC catalyzed by ACC synthase. The conversion of ACC to ethylene is catalyzed by ethylene-forming enzyme (EFE), which is, barring a few exceptions, constitutively expressed in most higher plants [29, 53]. But, generally, ethylene biosynthesis appears to be regulated by both ACC synthase and EFE activities depending upon the plant tissue and its physiological state.

Another facet of fruit ripening is the inverse developmental relationship seen between the timing of fruit's ability to produce ethylene and the accumulation of polyamines [30]. A similar reciprocal relationship between polyamines and ethylene has been invoked during the development of somatic embryos in cell cultures [33]. Both ethylene and polyamines share SAM as a common precursor and methylthioadenosine as a common byproduct [27, 53]. Tomato cultivars that accumulate and maintain relatively high levels of polyamines have been found to produce relatively low levels of ethylene and to have longer shelf-life [11, 42]. Furthermore, exogenously applied polyamines inhibit the plant's ability to produce ethylene [3, 5, 14, 48], perhaps by suppressing the induction of ACC synthase activity [28]. Conversely, ethylene suppresses the accumulation of polyamines [4]. This reciprocal inhibition of ethylene biosynthesis by polyamines, and vice versa, appears to be concentration-dependent. Although there is considerable literature [13] on the effects of polyamines in plant growth and development, there is very little information on their mode of action at the molecular level.

Since the manipulation of ethylene levels in crops has many possible applications, the molecular mechanisms that underly the activation and repression of the ACC synthase gene(s) are of considerable interest. The cloning and sequencing of its mRNA from wound-induced zucchini [40, 41], tomato [51] and squash [34] pericarp tissues have given a fresh, molecular perspective to investigations on the regulation of ethylene

production, in general, and ACC synthase, in particular. Notably, a winter squash ACC synthase cDNA clone has been used to demonstrate that autoinhibition of ethylene biosynthesis is accompanied by a suppression of ACC synthase gene transcription [34]. Similarly, the sequences of ACC synthase cDNA clones could be used to address questions on the molecular regulation of ACC synthase transcript level during ripening and by exogenously applied polyamines. Therefore, a 70 bp oligonucleotide with sequence similarity to wound-induced ACC synthase cDNA clones was synthesized. Using this oligonucleotide as a probe, we show that wounding results in the accumulation of an ACC synthase transcript. Furthermore, we demonstrate that both the induction of this transcript and ACC synthase activity during wounding of tomato pericarp tissue are blocked in the presence of salicylic acid, a known inhibitor of a wounding signal, and polyamines, the anti-senescence plant growth regulators.

Materials and methods

Plant material and wounding conditions

Lycopersicon esculentum cv. Pik-Red plants were grown either in a greenhouse under 16 h daylight and 8 h darkness at temperatures ranging from 20 to 30 °C or in the USDA fields at Beltsville, Maryland. The tomato fruits were harvested at mature green (green and firm), breaker (orange at blossom end and firm), early-red (red and firm) and red-ripe (red and soft) stages. Fruits were surface-sterilized with 70% ethanol, rinsed with distilled water, and sliced into 5 mm cubes. Some of the pericarp tissue was frozen immediately in liquid N_2 (time zero control). The remaining slices were incubated in an isotonic medium containing 0.6 M sorbitol and 10 mM 2(N-morpholino)ethanesulfonic acid (MES)-KOH, pH 6.0 [26], under fluorescent white light (0.6 μE m^{-2} s^{-1}). At different times, samples were taken and frozen in liquid N_2.

Salicylic acid and polyamine treatments

The wounded tomato pericarp tissue from early-red tomato was divided into identical 590 ml flasks containing a thin layer (20 ml) of the isotonic medium. The fruit tissue was incubated with or without salicylic acid (final concentration 2 mM) or the polyamines putrescine, spermidine and spermine (each at 10 mM final concentration). The effective concentrations of salicylic acid and polyamines were initially determined from dose-response curves. Samples of tomato slices were removed after various periods of incubation and frozen in liquid N_2.

ACC synthase activity assay

The frozen tomato pericarp tissue was homogenized using a mortar and a pestle in a medium containing 100 mM [N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid (EPPS)-KOH pH 8.5, 0.4 mM dithiothreitol, 5 μM pyridoxal phosphate, and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin A). The homogenates were gel filtered on a G-25 Sephadex column and aliquots (0.5 ml) of the eluted protein were assayed for ACC synthase enzyme activity as previously described [31].

Extraction of nucleic acids from tomato

Tomato genomic DNA was isolated from expanded leaves as previously described [52]. Total RNA was isolated using a modified method [10]. Tomato tissue was homogenized in a solution of 6 M guanidine isothiocyanate, 0.5% sodium N-laurylsarcosine, 20 mM sodium citrate, 0.1 M 2-mercaptoethanol, and 0.1% antifoam A, pH 7.0. The homogenate was filtered through a single layer of miracloth (Calbiochem, La Jolla, CA) to remove particulate material. RNA in the filtrate was precipitated overnight by the addition of 0.6 volume of absolute ethanol and incubation at −20 °C. The pellet was resuspended in 50 mM

Tris-HCl, 5 mM EDTA, pH 7.5, and then extracted with phenol and chloroform. RNA in the aqueous phase was again precipitated using LiCl at a final concentration of 2 M at 4 °C overnight. RNA was recovered by centrifugation at 12 000 × g for 20 min, resuspended in a solution containing 40 mM Tris-HCl, pH 7.5, 10 mM sodium chloride, 6 mM magnesium chloride, 10 mM dithiothreitol, and 40 000 units/ml RNA-sin (Promega Biotec, Madison, WI) and then incubated at 37 °C for 30 min with 35 units/ml of RQ1 RNase-free DNase (Promega Biotec). The RNA was re-extracted with phenol and chloroform, resuspended in 50 μl of diethylpyrocarbonate-treated water and quantified by measuring its absorbance at 260 nm.

Radiolabeling of the 70-mer oligonucleotide probe by PCR

Two oligonucleotides were synthesized, based on published ACC synthase gene sequences that represent amino acid sequences from 270 to 293 residues [51]. A 42-mer (sense strand: 5'-TTAGT TCACA TCGTC TACAG TCTTT CAAAA GACAT GGGGT TA-3') and a 40-mer (antisense strand: 5'-AATAT ATGAT TCCGA CTCTA AATCC TGGTA ACCCC ATGTC-3'), sharing a complementary sequence of 12 bases (underlined), were prepared using an Applied Biosystem model 380A DNA synthesizer. The polymerase chain reaction (PCR) was used for extension of the partial duplex formed by annealing the two primers. Each reaction contained 5 pmol of each of the two primers, 10 000 pmol of dGTP, dCTP and dTTP, and 83 pmol of [α-^{32}P]dATP (6000 Ci/mmol), 1.25 U of the Perkin-Elmer-Cetus AmpliTaq DNA polymerase, and a modified form of *Thermus aquaticus* DNA polymerase [20], in a buffer provided by Perkin-Elmer-Cetus and was run for 20 cycles of 94 °C (1 min) −45 °C (1 min) −72 °C (1 min). The radio-labeled 70 bp oligonucleotide was separated from the unincorporated free [α-^{32}P]dATP by electrophoresis on a 3% low-melting agarose gel using TAE buffer (40 mM Tris-acetate, 1 mM

EDTA, pH 8.0) at 2 V/cm for 2 h. The radiolabeled band was excised and melted at 95 °C for 15 min to denature the double-stranded 70-mer and then mixed with 3 ml of hybridization buffer and used for Southern (47) or northern hybridization analysis.

Preparation of radioactively labeled nucleic acid probes

The glycine-rich protein cDNA clone, previously also called pT53, and rRNA probes were labeled in *in vitro* transcription reactions as previously described [36]. Each reaction containing 40 mM Tris-HCl pH 7.5, 6 mM magnesium chloride, 2 mM spermidine, 10 mM sodium chloride, 10 mM dithiothreitol, 20 units of RNAsin, 2.5 mM ATP, GTP and UTP, 100 μCi (400 Ci/mmol) of [α-^{32}P] CTP, and 10 units of T7 RNA Polymerase (United States Biochemical Corporation, Cleveland, OH) was incubated for 1 h at 37 °C. The DNA template was then digested with RQ1 RNAse-free DNAse.

Southern and northern blot analysis

Genomic DNA (10 μg) was digested using various restriction endonucleases, separated by electrophoresis on 0.7% agarose gels [25], and transferred to a nylon membrane (Biodyne A, Pall Ultrafine Filtration, Glen Cove, NY) as described by Southern [47]. For the northern blots, total RNA (5 to 25 μg) was fractionated on formaldehyde-agarose gels [25] and transferred to the nylon membranes using 10 × SSC (1.5 M NaCl, 0.15 M citric acid). The nucleic acids were cross-linked to the nylon membrane by ultraviolet irradiation at 1200 μJ (Stratalinker) and then baking at 80 °C in vacuum for 1 h. The blots were prehybridized in a solution of 6 × SSC, 50 mM sodium phosphate pH 6.8, 5 × Denhardt's solution, 0.1% SDS, 2 mM EDTA, 0.2 mg/ml denatured salmon sperm DNA and 0.2 mg/ml yeast tRNA.

All hybridizations were carried out at 42 °C. The blots were washed in 2 × SSC once at room temperature and once in 2 × SSC containing 0.1% SDS at 42 °C; each wash was for 30 min. Final wash was given in 0.2 × SSC containing 0.1% SDS at 48 °C for 30 min. After drying, the blots were exposed to X-ray films with intensifying screen at −80 °C.

Each of the northern blot experiments was repeated three times. Although fruit-to-fruit variability was evident, as alluded to previously [36], patterns presented here were reproducible.

Results

Specificity of the wound-induced ACC synthase gene probe

Our strategy was to use a relatively large oligonucleotide probe showing a high sequence similarity to both the tomato [51] and squash [34] wound-inducible ACC synthase cDNAs, in order to study the regulation of the tomato ACC synthase transcript. We synthesized two oligonucleotides (one 42 bases long from the sense strand and the other 40 bases long from the antisense strand), with a complementary stretch of 12 bp at the 3' ends. The short, double-strand DNA duplex with the two free 3' ends served as a primer for DNA polymerase to fill in the remaining regions thereby generating a double-stranded DNA segment of 70 bp (70-mer). This 70 bp DNA sequence corresponds to the highly conserved, pyridoxal phosphate-binding site region of the ACC synthase protein [54]. Initial experiments were performed to confirm that the 70-mer does, in fact, hybridize specifically to tomato ACC synthase sequences. The 70-mer oligonucleotide probe was radiolabeled with [α-^{32}P] dATP using PCR as described in Materials and methods, then its hybridization to tomato DNA restriction fragments and total tomato pericarp RNA was analyzed.

Genomic DNA from tomato fruit was digested to completion with one of several restriction endonucleases (*Hind* III, *Eco* RI, *Bam* HI, *Acc* I and *Bgl* II), fractionated on agarose gels, then transferred to nylon membranes and hybridized

with the ^{32}P-labeled 70-mer. Results presented in Fig. 1 show hybridization of the 70-mer to tomato DNA fragments of 1.3 kb (lane 2), 3 kb (lane 3), 30 kb (lane 4) and 7 kb (lane 5) generated by *Hin*d III, *Eco* RI, *Bam* HI and *Acc* I, respectively. The probe also hybridized to a 4.1 kb *Bgl* II fragment (data not shown). Hybridization to the 3 kb *Eco* RI and the 4.1 kb *Bgl* II fragments is consistent with published results [51]. Strong hybridization between the labeled 70-mer and a wound-inducible transcript of 1.8 kb was also detected on northern blots (Figs. 3 and 5). Significant increase in the transcript level occurred between 1 and 4 h after wounding reaching a maximum at

Fig. 1. Southern blot analysis of the wound-inducible ACC synthase gene. Tomato genomic DNA (10 μg) was digested to completion by the indicated restriction endonucleases: *Hin*d III, *Eco* RI, *Bam* HI and *Acc* I. Each digest was fractionated on a 0.7% agarose gel, transferred to a nylon membrane, UV-cross-linked, and then hybridized with the [α-^{32}P]dATP-labeled 70 bp oligonucleotide. The blot was then washed at 50 °C in 0.2 × SSC, 0.1% SDS for 1 h and exposed to Kodak RP5 X-ray film at −80 °C for 1 week. Lane 1 shows *Hin*d III-digested λ phage DNA markers.

8 h in early-red fruit (data not shown). Since 1.8 kb is the size reported for the tomato, winter squash and zucchini ACC synthase transcript, which is also wound-inducible, these results indicate that the 70-mer probe is hybridizing specifically to ACC synthase sequences. Therefore, throughout this study, we used the 70-mer as a probe to study the regulation of ACC synthase transcript level.

Developmental and organ-specific regulation

Although accumulation of the 1.8 kb ACC synthase transcript was considerable in wounded fruit consistent with induction of this enzyme during these conditions [18, 19, 28], a basal level of the transcript was apparent in the unwounded control tissues, much more appeared to be present in the early-red stage fruit than in the red-ripe stage (data not shown). This prompted us to check if the 70-mer probe hybridizes to a similar transcript in fruit at different stages of ripening and in other organs of the plant. To address this question, total RNA isolated from unwounded green, breaker, early-red and red-ripe stages of fruit, as well as from tomato leaf, stem and root tissues was subjected to northern blot analysis. The results are presented in Fig. 2. Indeed, a 1.8 kb ACC synthase transcript is evident. The highest transcript level was found in the early-red tomato fruit tissue that produces highest rates of ethylene. In contrast to the fruit tissue, only low ACC synthase transcript levels were seen in leaf, stem and root tissue. Hybridization to an additional band at 1.25 kb was noted in root samples (Fig. 2R) and in 16 h wounded fruit samples (data not shown). This hybridization may represent another, late transcript or a product of degradation of the 1.8 kb transcript. These differences in the levels of the 1.8 kb ACC synthase transcript between the fruit and the other tomato tissues correlate with the different rates of ethylene production associated with them: the ethylene production rate in leaf, stem and root tissues is lower by an order of magnitude or more compared to the ripening fruit.
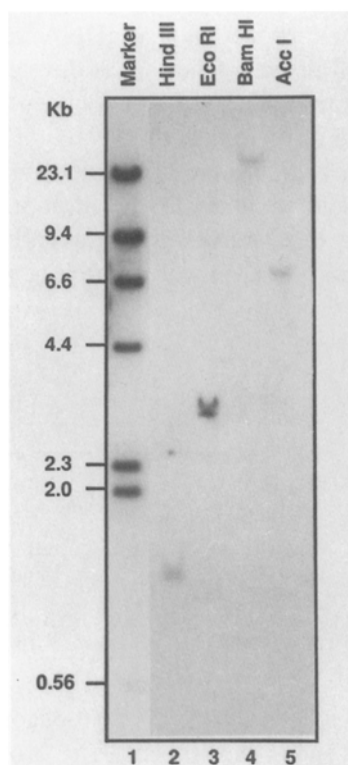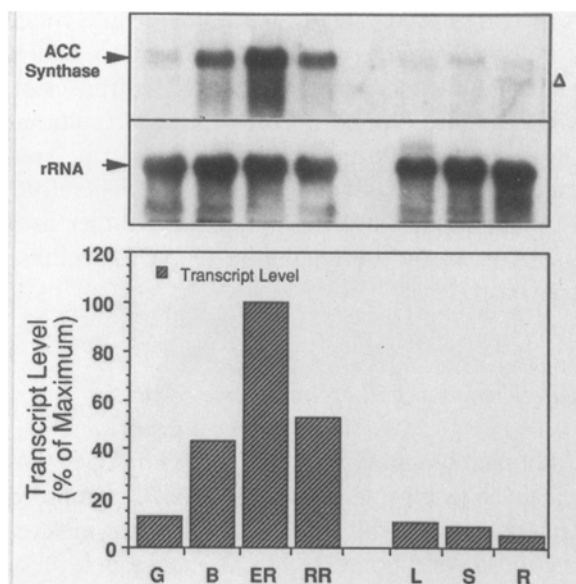
Fig. 2. Developmental and organ-specific regulation of the wound-inducible ACC synthase transcript. Total RNA (5 μg) isolated from unwounded green (G), breaker (B), early-red (ER) and red-ripe (RR) tomato fruit, and from tomato leaf (L), stem (S) and root (R) tissue was fractionated on formaldehyde-containing 1% agarose gels. The RNAs were transferred to nylon membranes, cross-linked by UV irradiation, then hybridized first to the 70-mer ACC synthase probe (5.0×10^9 cpm/μg), and later to a rRNA probe (northern blots). The blots were exposed to an X-ray film for 1 week at −80 °C. The signals on the X-ray films were quantified densitometrically and the ACC synthase transcript level normalized to the rRNA level in each sample. The quantified data are plotted as a % of maximum transcript level (hatched bars). The open triangle indicates hybridization to a 1.25 kb transcript.

Inhibition of wound-regulated ACC synthase activity and transcript accumulation by salicylic acid

Salicylic acid is a naturally occurring chemical that inhibits EFE [21] and the wounding signal in tomato plants [12]. However, little is known about its regulation of ACC synthase activity and ACC synthase transcript level during wounding. This natural compound might also inhibit the wound induction of ACC synthase activity and/or its transcript. To address this question, tomato pericarp tissue was wounded and incubated for 9 h in the presence or absence of 2 mM salicylic

acid. Specific activity of extractable ACC synthase in 9 h sample wounded in the presence of salicylic acid was found to be 0.24 nmol/h per mg protein compared to 2.55 nmol/h per mg protein in the control samples (wounded in the absence of salicylic acid), which amounted to about 90% inhibition in the induction of the enzyme activity.

Similarly, the effect of salicylic acid on the wound induction of the ACC synthase transcript was determined in fruit tissues incubated in the presence or absence of this chemical. The results in Fig. 3 show that salicylic acid also inhibits the wound-induced increase in the ACC synthase transcript level. The specificity of this effect on the ACC synthase transcript was tested by hybridizing the northern blot separately to two additional probes. One probe was a cDNA clone of a glycine-rich protein gene that is developmentally regulated [36] while the other probe is for rRNA. Results in Fig. 3 confirm that by 6 h of wounding the glycine-rich protein transcript is repressed, and further that salicylic acid addition does not prevent or accelerate this repression. Furthermore, the rRNA level was much less affected by
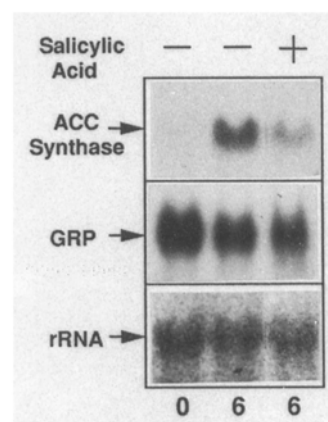

Fig. 3. Effect of salicylic acid on wound-regulated ACC synthase and glycine-rich protein transcripts. Early-red tomato pericarp tissue was wounded and incubated for 6 h in the absence (−) or presence (+) of 2 mM salicylic acid. Total RNA (20 μg/sample) was isolated from unwounded control (0 h of wounding) and 6 h wounded tissues, gel electrophoresed, blotted and then hybridized with the 70-mer probe. The blot was stripped and subsequently reprobed with a glycine-rich protein cRNA probe and a rRNA probe.

wounding or treatment with salicylic acid. There-fore, salicylic acid has a specific effect on the accumulation of the wound-induced ACC syn-thase transcript. In addition, these results suggest that the wound signal regulating ACC synthase transcript level is perhaps similar to that for other wound-responsive genes, such as the proteinase inhibitor [12]. Although ethylene may mediate some of the wound-related plant responses, we have not determined its involvement in the regu-lation of wound induction of ACC synthase (see also Discussion).

Polyamines inhibit the wound-induced increases in ACC synthase transcript level and enzyme activity

Polyamines have been implicated in delaying rip-ening and senescence processes [15, 45] and shown to inhibit the development of ACC syn-thase activity and/or ethylene production (see In-troduction). This raises the question of whether the polyamine effect involves the regulation of the ACC synthase transcript level. In order to ad-dress this question, we incubated early-red to-mato slices in the isotonic medium (see Materi-als and methods) containing either putrescine, spermidine or spermine from 1 to 10 h. First, cell-free extracts were prepared to determine whether or not the production of ACC synthase activity upon wounding was inhibited by these poly-amines.

The results presented in Fig. 4 confirm that wounding of tomato results in a continuous in-crease in the activity of ACC synthase as ex-pected (open bars). The addition of putrescine, spermidine or spermine during the wounding pro-cess inhibited this induction; however, different polyamines had slightly different effects on the production of ACC synthase. For instance, the inhibitory effects of putrescine and spermidine were transient and lasted only until 6 h after wounding. By 10 h after wounding, the putrescine effect was completely reversed while that of sper-midine was partially reversed. In contrast, the inhibitory effect of spermine was sustained throughout the wounding period (Fig. 4). These
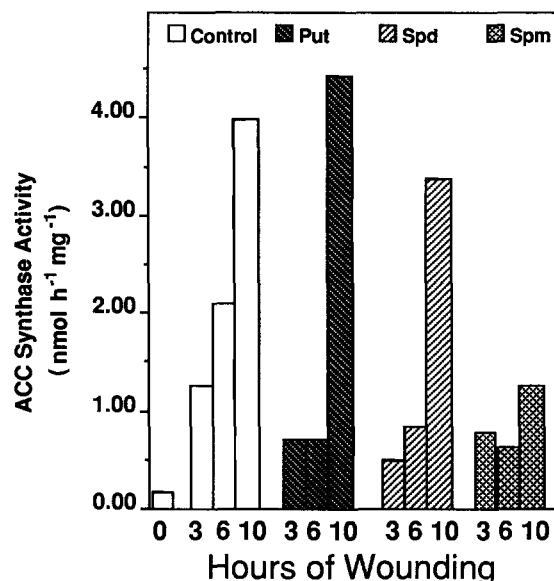

Fig. 4. Inhibition of ACC synthase enzyme activity by poly-amines. Early-red tomato pericarp tissue was wounded and incubated separately for 0, 3, 6 and 10 h in the absence (Con-trol) or presence of 10 mM putrescine (Put), spermidine (Spd), or spermine (Spm). Each sample was homogenized and cell-free extracts were prepared for determination of ACC syn-thase enzyme activity as described in Materials and methods.

results are consistent with previous reports show-ing that spermine has a stronger inhibitory effect on ethylene production than other polyamines [3, 28, 38].

The levels of the wound-inducible ACC syn-thase transcript in samples incubated for 1 to 6 h following wounding in the presence or absence of polyamines were analyzed on northern blots. The results (Fig. 5) show that the different polyamines inhibited the accumulation of the wound-induc-ible ACC synthase transcript in wounded peri-carp tissue to different degrees. By 3 h, the tran-script level dropped to approximately 65, 70 and 35% of the control in the presence of putrescine, spermidine and spermine, respectively. But upon further incubation (6 h), the ACC synthase tran-script accumulated to the control levels in putrescine- and spermidine-treated tissues. In spermine-treated tissues, however, the transcript level was further diminished to 45% of the con-trol by 6 h. Putrescine and spermidine effects on
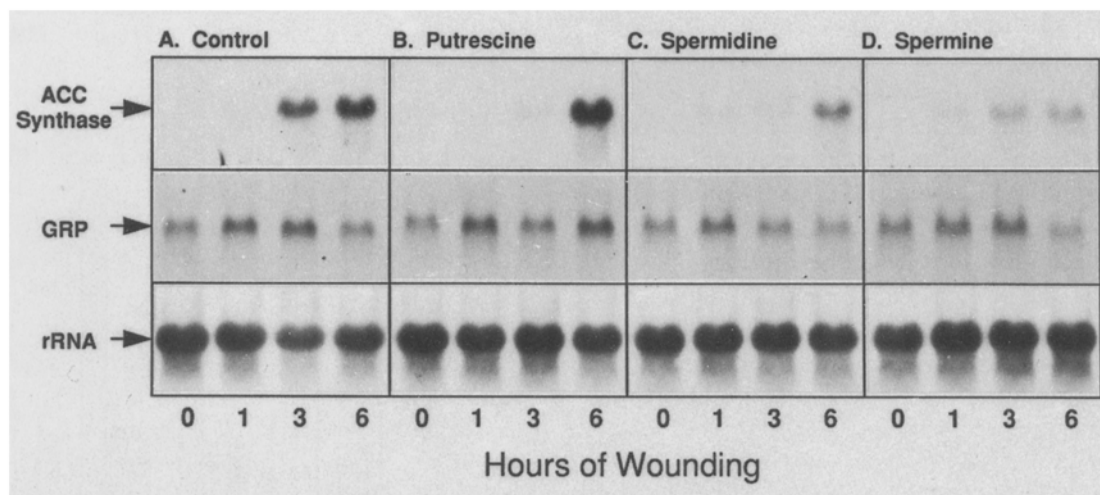
Fig. 5. Inhibition of the wound induction of the ACC synthase transcript by polyamines. Early-red tomato pericarp tissue was wounded and incubated separately for 0, 1, 3, and 6 h in the absence (Control) or presence of 10 mM putrescine, spermidine, or spermine. Total RNA from each sample was isolated, and analyzed (20 μg/lane) by northern blot hybridization using the 70-mer probe, glycine-rich protein cRNA probe, and the rRNA probe.

the ACC synthase transcript level are consistent with their effects on the development of ACC synthase activity upon wounding (Fig. 4). However, it is evident that the increased transcript levels in putrescine- and spermidine-treated tissues on longer incubation, which approached control levels, preceded the observed rise in the enzyme activity (compare 6 h time points in Fig. 5 and Fig. 4). These results suggest that the two processes are linked.

The specificity of polyamines' effect on the ACC synthase transcript level was tested by hybridizing the blots separately to probes for a glycine-rich protein gene and a rRNA. The results in Fig. 5 show that the pattern of the glycine-rich protein transcript upon wounding is not markedly affected in the tissues incubated with different polyamines; at best, spermidine and spermine appear to accentuate the trend seen in the wounded control tissue (Fig. 5A). Furthermore, the rRNA level was not affected by either wounding or treatment of tissue with polyamines. Taken together, these results implicate polyamines as regulators of wound-induced ACC synthase transcript.

Discussion

ACC synthase is a key, rate-limiting enzyme regulating the production of ethylene in higher plants. The induction of this enzyme upon wounding of winter squash [34] and tomato [51] has been shown to involve induction of a specific transcript. The wound-inducible ACC synthase gene in tomato as well as in winter squash was estimated to be encoded by a single copy in the genome. However, two different ACC synthase cDNAs were cloned from tomato pericarp tissue [51]. Moreover, recent results suggest that several (putative) gene(s) for ACC synthase may be present in the tomato genome (A. Theologis, personal communication). These results together with reports of multiple forms of ACC synthase [30] would indicate the presence of more than one type of ACC synthase, at least in tomato fruit.

The 70-mer probe used in our experiments hybridized to 1.8 kb transcript. Occasional and weak hybridization to a 1.25 kb RNA was also observed. Perhaps this is a degradation product of the 1.8 kb transcript or represents another ACC synthase gene transcript. The 1.8 kb transcript is

clearly induced by wounding and a similar size transcript is induced during ripening. Whether the same ACC synthase transcript is induced by wounding and during ripening can not be determined without using gene-specific probes. However, while this paper was in preparation, Olson et al. [35] reported that an ACC synthase transcript that is 200 bp shorter than the wound-inducible transcript is differentially expressed. The 70-mer probe used in our study is 76% similar to the homologous region of the cDNA sequenced by Olson et al. [35] and hybridizes to a 1.8 kb transcript. Our results with the wound-inducible ACC synthase confirm and extend the observations of Olson et al. [35], in that the wound-inducible transcript may also be developmentally regulated, and may also be present in the leaves, stem and roots of tomato plant albeit at a much lower level. Future experiments using gene-specific probes should help in resolving these questions.

A marked decrease in the 1.8 kb transcript level occurred in the presence of salicylic acid, which correlated with a strong inhibition in the production of ACC synthase activity upon wounding. Salicylic acid is considered as a transduction signal for the induction of PR proteins [24, 32]. In tomato plants, however, salicylic acid is also known to inhibit wound signal transduction [12]. Our results with salicylic acid are similar to those found for the systemic accumulation of protein-ase inhibitor proteins, suggesting that a similar wound signal might be involved in the induction of ACC synthase. The inhibitory effects of salicylic acid on both the ACC synthase transcript level and enzyme activity are novel.

We have demonstrated that polyamines inhibit the wound induction of both ACC synthase activity and its transcript level. This inhibition is characterized by three things: (1) both the ACC synthase enzyme activity and its transcript are coordinately inhibited, the effect on the transcript level being realized before that on the enzyme activity; (2) each of the polyamines, putrescine, spermidine and spermine, was an effective inhibitor, the most effective being spermine; and (3) the polyamine-mediated inhibition was transient in

nature. The transient nature of the polyamine-mediated inhibition could be explained in a number of ways. Some of the possibilities include sequestration of polyamines in the cell at sites away from the target, oxidation of polyamines and thus lowering their effective concentration [43, 46], or that prolonged wounding results in switching on a mechanism that may override the effect of the polyamines. One can only speculate on the transduction pathway through which polyamines mediate regulation of ACC synthase expression. Our results on the ineffectiveness of polyamines to modulate the glycine-rich protein transcript, which is developmentally expressed and wound-regulated [36], suggest that the polyamine effect on wound-inducible ACC synthase transcript is relatively specific. The results clearly demonstrate for the first time that polyamines have the potential to regulate ethylene biosynthesis by affecting the accumulation of the ACC synthase transcript and add strength to the hypothesis that high endogenous levels of polyamines in growing tissues keep the ACC synthase gene(s) repressed and thus inhibit the untimely production of ethylene during early development.

At this time it is not known whether the increase in ACC synthase transcript level results from an increase in transcription rate or from an increase in mRNA stability, or from both. The expression of the typical wound-inducible protease inhibitor (E17) gene is known to be regulated by both transcriptional and posttranscriptional events [23]. Nuclear run-on transcription assays need to be performed with tissues at different developmental stages, at different times of wounding and in the presence of different concentrations of ethylene using gene-specific probes to differentiate between the various possible mechanisms involved in the regulation of ACC synthase gene expression.

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